LIPOSOME-MEDIATED RICIN TOXICITY IN RICIN-RESISTANT CELLS

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1. Introduction

The mechanism of entry of the toxic lectin ricin into the cell cytoplasm is poorly understood. It is known that ricin, via the B chain, must first bind to galactose sugar residues at the cell surface, where it is taken up, probably by endocytosis, into the cytoplasm allowing the toxic A chain to arrest protein synthesis by inactivation of the 60 S ribosomal subunit [1,2].

Cells have been isolated which are resistant to the toxic effects of ricin in vivo [3,4]. In particular a clone of BHK cells, Ric^R 14, lacks ricin receptors at the cell surface, but is sensitive to the toxic effects of ricin when assayed by cell-free methods [5]. This cell line would therefore be a useful indicator to explore further the mechanism of ricin action if other methods of introducing ricin to the cell could be employed. Some success has been achieved in introducing ricin into mouse macrophages via their Fc receptors [6]. However, a more general method of introducing biologically active molecules into the cell cytoplasm via liposomes has been developed [7].

Negatively-charged liposomes that are fluid at 37°C have been incorporated into cultured cells by fusion with the cell membrane [8] and the biological activity of liposome-entrapped material demonstrated [9,10]. We describe here the preparation of liposomes containing ricin and their effect on protein synthesis after fusion of liposomes with normal and ricin-resistant cells.

2. Materials and methods

Egg phosphatidyl choline and beef brain phosphatidyl serine were obtained from Lipid Products, Redhill, Surrey. Cholesterol was from Sigma Chemical Co., Poole, Dorset; Ricin from Miles Labs, Stoke Poges; Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala. Glasgow Modified Minimal Essential Medium and foetal calf serum were from Gibco Biocult, Paisley. Na ^{125}I carrier free (100 mCi/ml) and [^{3}H]leucine (55 Ci/mmol) were purchased from Radiochemical Centre, Amersham, Bucks.

2.1. *Preparation of liposomes containing ricin*

Liposomes (20 μmol lipid/ml) were prepared by evaporating chloroform solutions of phosphatidyl choline, phosphatidyl serine and cholesterol, in molar ratios 9:1:10 to dryness under vacuum. The film of lipids was suspended in 1 ml 5 mM phosphate buffer (pH 7.2), containing 0.2 M NaCl with or without 1 mg ricin. Trace amounts of ^{125}I-labelled ricin [11] were added to quantitate ricin capture inside vesicles. The suspension was vortexed for 30 min at 37°C and sonicated under nitrogen in a bath-type sonicator at 34–37°C for 60 min. The liposomes were then equilibrated for 60 min at room temperature before gel chromatography on a Sephadex G-200 column (1.1 × 31 cm) equilibrated in the above phosphate buffer at room temperature. ^{125}I radioactivity was measured in a Packard-5230 Auto gamma scintillation spectrometer.

2.2. *Interaction of liposomes with cells*

BHK and Ric^R 14 cells were grown [3,4] to near confluency on 35 mm diam. tissue culture dishes (1×10^6 cells/dish) at 36°C in Glasgow Modified Minimal Essential Medium containing 10% (v/v) foetal calf serum. After washing with phosphate-buffered saline [4], dilutions of empty liposomes or liposomes containing ricin prepared as above were

added to BHK cells and Ric^R 14 cells, and incubated at 36°C for 2 h. The cells were washed in phosphate-buffered saline and then incubated in a solution of [^{3}H]leucine (1 μCi/ml) [12] at 36°C for 45 min. The cell monolayer was washed with phosphate-buffered saline and fixed in situ with two washes of 10% (v/v) perchloric acid–2% (w/v) phosphotungstic acid and twice with ice cold ethanol. Dry monolayers were dissolved in 0.5 N NaOH (1 ml) and aliquots taken for protein and ^{3}H radioactivity determinations [12].

In some experiments cells became detached from the dish after incubation with liposomes, in which case cells were centrifuged, incubated with [^{3}H]leucine in solution and after adding NaOH to 0.1 N for 20 min at 37°C, protein material was precipitated with 25% (w/v) trichloroacetic acid and collected on Whatman GF/C filters for ^{3}H radioactivity analysis. Both methods of assessment of incorporation of [^{3}H]leucine yielded identical results.

Sensitivity to solutions of ricin was determined by washing cells as above and adding ricin solutions in phosphate-buffered saline, in concentrations indicated, to the cell monolayer.

3. Results and discussion

We have found that ricin is very efficiently incorporated into liposomes after sonication of lipid and ricin mixtures. In some experiments as much as 0.025 mg ricin/μmol lipid becomes associated with the liposomes and, as fig.1A shows, Sephadex gel chromatography separates trapped from non-trapped material. Since ricin is anionic at pH 7.2 (pI 7.1) [13], the conditions of liposome preparation exclude the possibility of electrostatic interaction of ricin with the negatively charged liposomes. Mixing of free ricin with preformed liposomes, after sonication, does not lead to incorporation as all the radioactivity is retained by the Sephadex column upon gel chromatography (fig.1B). Furthermore, when liposomes containing ricin are treated with low concentrations (1%) of the non-ionic detergent Triton X-100 > 80% of the recoverable radioactivity is released – further evidence against electrostatic association [14]. Liposomes containing ricin are extremely stable over a wide variety of conditions and no leakage of incorporated ricin is observed even after incubation of the

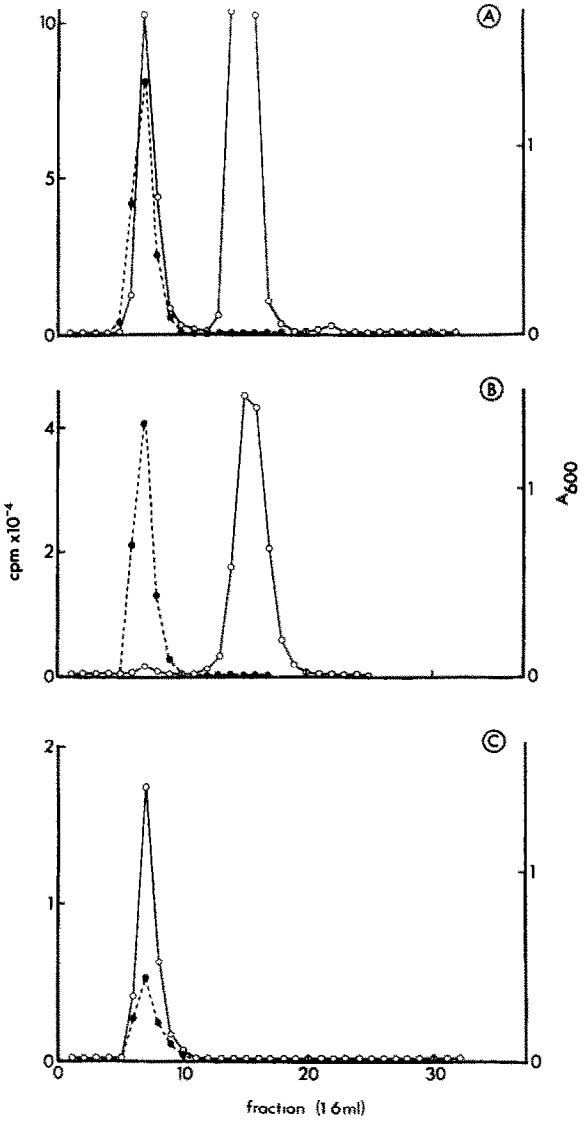

Fig.1. Gel chromatography of liposomes: Liposomes were prepared as in section 2 and chromatographed on a Sephadex G-200 column (1.1 × 31 cm). Column fractions (1.6 ml) were collected at a flow rate of 7.5 ml/h at an operating pressure of 20 cm water. ^{125}I radioactivity (○——○) was measured and A_{600} (●– – –●) determined for each fraction. (A) Elution of liposomes containing ^{125}I-labelled ricin from Sephadex G-200. (B) Elution of a mixture of preformed empty liposomes and ^{125}I-labelled ricin from Sephadex G-200. Empty liposomes were incubated for 3 h at 37°C with ^{125}I-labelled ricin before applying to the column. (C) A portion of pooled liposomes containing ^{125}I-labelled ricin fractions from fig.1A (fractions 6–8), were incubated at 36°C for 48 h and then re-chromatographed.

liposomes for 48 h at 36°C (fig.1C). Although it cannot be ruled out that some hydrophobic attraction of ricin within the lipid bilayer can occur, ricin is not superficially associated with the liposome surface. Agarose (Sepharose 4B) gel chromatography of liposomes containing ricin yields full recovery of applied lipid and protein material in fractions eluted with buffered saline (data not shown). This would indicate that the galactose binding sites of ricin are not externally orientated in the liposome, as agarose is an efficient affinity adsorbent for this lectin [15].

Figure 2 shows the effect of liposomes containing ricin, of empty liposomes and of ricin at different concentrations on BHK cells or on Ric^R 14 cells, a genetically stable clone of BHK cells which is resistant in vivo to ricin toxicity [3,4]. When liposomes containing ricin are incubated with BHK or Ric^R 14 cells, protein synthesis in both types of cells, as measured by the incorporation of [^{3}H]leucine, is inhibited (fig.2). Conversely, when free ricin is added at the same concentrations as ricin entrapped in liposomes, BHK cell protein synthesis is inhibited to $>90\%$ at very low levels (>5 μg/ml), whereas Ric^R 14 cells even at concentrations as high as 60 μg ricin/ml show only 50% inhibition in [^{3}H]leucine incorporation (fig.2). Empty liposomes have no effect on protein synthesis of BHK and Ric^R 14 cells (fig.2).

It is well established that Ric^R 14 cells lack ricin binding sites at the cell surface (over 90% reduction compared to BHK cells), due to a deficiency in sugar transferase activity regulating the expression of ricin binding carbohydrate sequences [3,4]. These cells are not blocked in any endocytotic mechanism since neuraminidase treatment which exposes new ricin binding carbohydrate sequences leads to ricin entry into the cells resulting in inhibition of protein synthesis [12,16]. The present results show that ricin can inhibit protein synthesis in ricin-resistant Ric^R 14 cells if a mechanism of introduction of the ricin into the cytoplasm other than those of exposed membrane receptors and endocytosis is used. Using negatively-charged liposomes, which are able to fuse with the cell membrane and release their contents into the cell cytoplasm, we have demonstrated that the resistance of Ric^R 14 cells does not reside in the protein synthesising machinery. Inadequate toxin binding to the membrane or reduced uptake or transport to the ribosomes or a combination of these

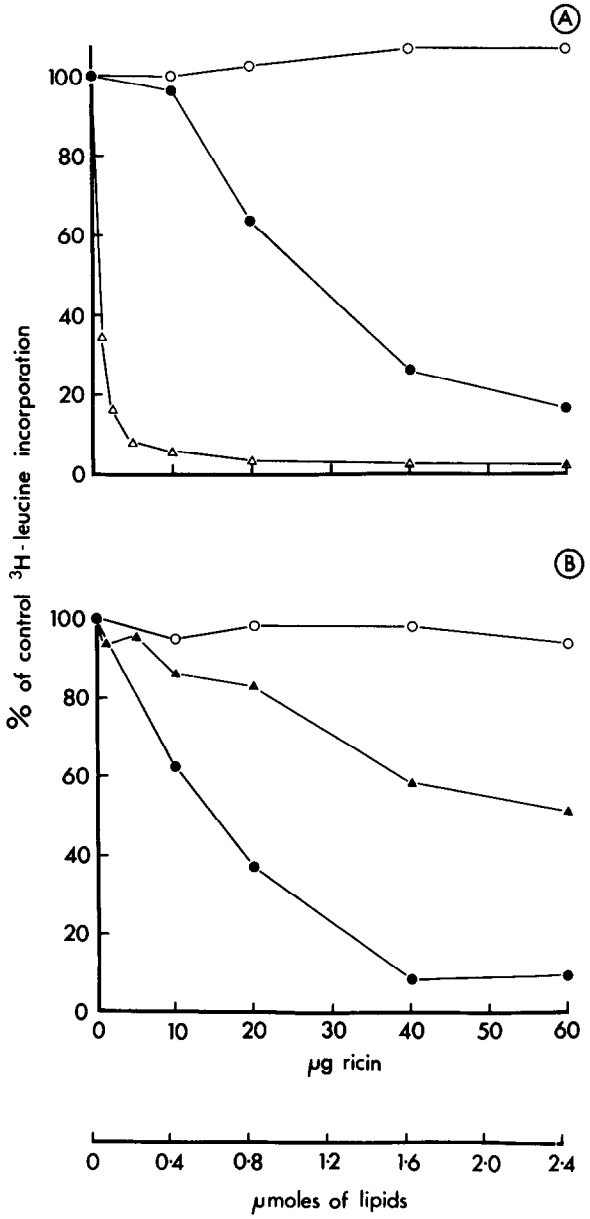

Fig.2. The effect of liposome and ricin treatment on BHK and Ric^R 14 cell protein synthesis: BHK (fig.2A) and Ric^R 14 (fig.2B) cells were incubated for 2 h at 36°C with either empty liposomes (o——o), liposomes containing ricin (•——•) or free ricin (△——△, ▲——▲), after which [^{3}H]leucine radioactivity incorporation into acid-precipitable material was assessed as in section 2. Specific activities, cpm/mg cell protein or cpm/10^6 cells for each dilution of liposomes or ricin are expressed as a percentage of phosphate-buffered saline control dishes.

factors, are responsible for resistance in these cells. In conclusion we believe that entrapping of ricin into liposomes would be a useful tool for the further analysis of the genetic lesions in ricin-resistant cells and for the selection of mutant cells in which protein synthesis is not sensitive to ricin. As ricin has anti-tumour properties [17], liposomes containing ricin may be used against cancer cells particularly if 'recognition ligands' could be included in the liposome membrane to induce preferential uptake of liposomes by tumour cells [18].

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